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Viable but Non-culturable State of Foodborne Bacteria: A Challenge in Food Safety

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Abstract
The viable but non-culturable (VBNC) is an odd adaptive condition which occur in responding of some bacteria to stressful conditions .1982 saw its initial detection. Unluckily, it has been documented that a number of foodborne pathogens can, during food processing and preservation; enter the VBNC state due to restricting environmental conditions. These conditions include extremely high or low temperatures, drying, high pressure, irradiation, pulsed electrical fields and the addition of preservatives and disinfectants. Foodborne pathogens provide a severe threat to public health and food safety once they reach the VBNC state since they are undetectable using standard plate count methods. This article overviews the different characteristics of the viable but non culturable (VBNC)state such as the biological characteristics, induction and resuscitation factors, and resuscitation mechanisms, as well as their relevance to food safety and public health.

Keywords: *VBNC, Foodborne pathogens, Induction, Resuscitation, Public Health significance, Food safety*

Introduction

The viable but non-culturable (VBNC) state was first identified and described by **Xu *et al.* (1982)** and was initially defined as a unique physiological condition. Bacteria cannot grow and remain active on standard culture media because they are exposed to certain environmental stresses. Similar to the VBNC state, dormancy is another nonculturable condition of bacteria that can be characterized operationally as a reversible state of metabolic shutdown **Kell *et al.* (1998). According to Mukamolova *et al.* (2003),** VBNC cells have detectable metabolic activity, which is not observed in dormant cells, so the VBNC state is portrayed slightly differently from dormancy. Nonetheless, a number of scientists believe that dormancy and the VBNC state are two distinct words for the same physiological state **Oliver, 2005; Ayrapetyan and Oliver (2016).**

Under harsh circumstances, a biologically dormant stage of life known as the VBNC state develops, which is imperceptible to traditional bacteriological techniques. On the other hand, according to **Schottroff *et al.* (2018),** VBNC bacteria maintain their detectable metabolic functionality, modest levels of gene expression and membrane integrity. Since VBNC cells cannot be cultured in traditional culture media like normal cells can, conventional detection techniques are useless for identifying bacterial infections in the VBNC state. As a result, there are difficulties in detecting infections. There are now 85 species of bacteria that can reach the VBNC state; 18 of which are non-pathogenic, and the remaining 67 are pathogenic. Certain foodborne pathogens continue to be virulent even after they enter the VBNC stage; this could be because, under some circumstances, they can be quickly revived into

culturable cells and exhibit pathogenicity **Li *et al.* (2014).**

The VBNC state depends on two elements that either stimulates the resuscitation process or the VBNC state itself, and it may be independent of the bacteria's capacity to resuscitate. Additionally, not all VBNC strains can undergo resuscitation, and the outcomes of recovery techniques vary depending on the strain **Kan *et al.* (2019).** Foodborne illness, which can include vomiting, diarrhoea, and damage to the kidneys and liver, has become a serious concern worldwide. Dormancy and the VBNC state have presented difficulties for traditional culture-based methods of counting microorganisms and assessing the vitality of bacterial cells through colony formation **Gao *et al.* (2021).** The possibility that VBNC bacteria can infect people highlights how important the VBNC condition is for both public health and food safety **Ashbolt, (2015).**

Although it is usually believed that VBNC pathogens cannot cause diseases, the virulence of VBNC pathogens can be sustained or regained following resuscitation, resulting in infection or disease **Du *et al.* (2007b); Nicolò and Guglielmino (2012).** By incubating with embryonated eggs, as an example, the *Listeria monocytogenes* VBNC cells regained virulence equivalent to that of culturable cells **Cappelier *et al.* (2007).** In this respect, **Tolba *et al.* (2020)** induced a VBNC state for a field strain of *L. monocytogenes* in vitro using different chemical antimicrobials of different concentrations. This organism was resuscitated and regained its activity after treatment with BHI broth with different percentage depending on the type and concentration of the chemical used.

Additionally, plenty of data suggests that VBNC pathogens could play a role in foodborne outbreaks. As an example, there was a foodborne illness outbreak in Japan that was linked to salted salmon contaminated with *Enterohemorrhagic Escherichia coli* O157. A variety of food products, such as vegetables, fruit juice **Nicolò *et al.* (2011),** Chicken **Chen *et al.* (2019)**, and Seafood **Zhang *et al.* (2019),** can harbor many microbial infections that can induce the VBNC condition. Furthermore, it has been reported that traditional disinfection techniques in water treatment systems may promote VBNC induction in addition to the detection of VBNC bacteria in drinking water **Zhang *et al.* (2018).** From an ecological point of view, it may be more sensible to consider the VBNC state as a common protective tactic for non-spore-forming bacteria.

Induction of VBNC state

The capacity of bacterial cells to withstand harsh environmental conditions is the primary feature of the VBNC state. Numerous physical and chemical stress factors are proposed to upset the natural balance of bacterial growth conditions and cause the bacteria to enter the VBNC state, even though extensive research is still needed to fully understand the effects of various conditions on VBNC induction **Li L *et al.* (2014).** Starvation **Gray *et al.* (2019),** extreme or low temperature **Wei and Zhao 2018),** osmotic **Wasfi *et al.* (2020)** and oxidative stress **Liao *et al.* (2020),** UV irradiation **Liao *et al.* (2018)**, pulsed light, pulsed electric field **Emanuel *et al.* (2021),** heavy metal **Maertens *et al.* (2021),** and acute alteration of pH or salinity **Wong and Wang (2004),** also food processing and preservation techniques **Li *et al.* (2020),** are the primary identified factors that induce bacterial stress and cause the bacteria to enter the VBNC state (Figure 1).

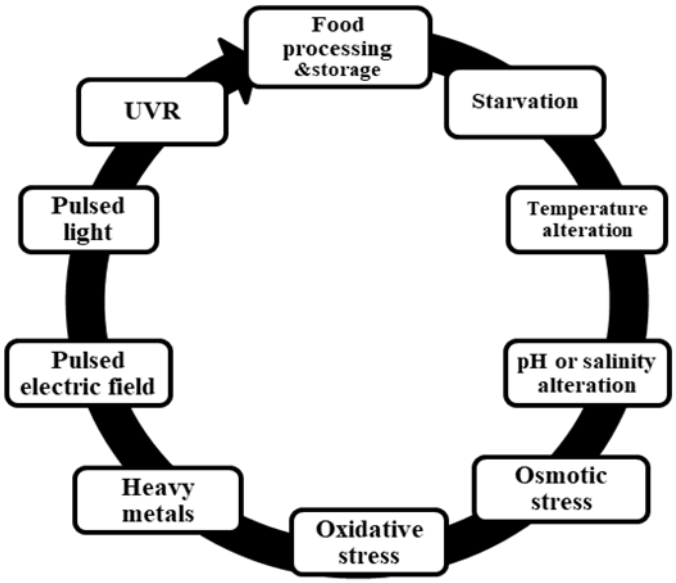

Fig. (1). Main stress factors inducing VBNC state

Despite the induction stress conditions for the VBNC state has been thoroughly examined, there has been limited focus on the molecular mechanisms and genetic regulation of the VBNC state. More strain-specific regulatory techniques are recommended due to the wide variety of VBNC bacterial species.

Characterization of VBNC bacteria

The viable but non-culturable bacteria (VBNC) exhibit specific genetic features and maintain their absorptive capacity in spite of losing their culture ability on standard media. These observations imply that culturable cells and VBNC cells share some characteristics. However, VBNC induction is responsible for several physiological changes, including a reduction in the absorption of nutrients, protein synthesis, as well as macromolecular metabolism **Zhao *et al.* (2017)**. In general, the VBNC state caused by considerable differences in cellular shape, metabolic activity, stress tolerance, and genetic regulation.

Cellular morphology

Rounding and dwarfing of the cell are the results of any morphological alteration that occurs during the shift towards the VBNC state. Furthermore, a feasible tactic in VBNC cells to limit the energy need is a reduction in the cell size **Progulske-Fox *et al.* (2022)**. Certain gram -positive bacteria, like *Enterococcus faecalis*, showed an increase in cell size in contrast to gram-negative bacteria **Signoretto *et al.* (2000).** Furthermore, **Robben *et al.* (2018)** report that gram-positive bacteria are more vulnerable to the induction of the VBNC condition. According to **Ierardi *et al.* (2020)**, *the Helicobacter pylori* VBNC cells maintained their virulence potential with low metabolic activity while having a coccoid morphology. . In the exponential phase, rod-shaped *Vibrio parahaemolyticus* also changes into a cocci form **Su *et al.* (2013).** These morphological alterations frequently occur in non VBNC cells as well; hence they do not indicate whether a bacterium is in the VBNC condition **Li *et al.* (2014).**

The Process of Metabolic Activity

In severe conditions, bacterial cells in the VBNC state continue to function metabolically **Du *et al.* (2007a).** In starvation, branched chain amino acids are the primary source of energy for VBNC bacteria **Ganesan *et al.* (2007).** However, deprived Vibrio cholera was shown to have decreased percentages of total lipids, carbohydrates, and poly-β-hydroxybutyrate, suggesting that these big molecules could serve as the main energy source for the cells to survive **Clements and Foster (1998)**. **Jeffreys *et al.* (1998)** reported that the VBNC cells also showed a decrease in DNA. Furthermore, **Trevors *et al.* (2012)** showed that the cytoplasm of VBNC and starving bacteria had fewer nucleic acid molecules

than normal bacteria. However, the mechanism behind the VBNC cells reduced DNA content is still unknown and requires more investigation. Despite sharing similarities with starved cells, VBNC cells have distinct amounts of protein expression.

Stress Tolerance

The VBNC cells exhibit higher levels of physical, chemical, and antibiotic resistance as compared to culturable cells. This could be attributed to their reduced metabolic activity and stronger cell wall, which is reinforced by enhanced peptidoglycan crosslinking **Signoretto *et al.* (2000).**

Nowakowska and Oliver (2013) created a model organism from *V. vulnificus* in order to investigate the VBNC condition. According to this model, *V. vulnificus* VBNC cells are resistant to a wide range of stimuli when they are dormant, such as hazardous heavy metals, high antibiotic dosages, high salinity, extreme heat, acid, and ethanol. Regarding chemical stresses, a related investigation on *V. parahaemolyticus* revealed that the VBNC cells were resistant to low salinity and H2O2, although they were still susceptible to bile salts **Wong and Wang (2004).** Many foodborne pathogens, including *Staph. aureus, V. vulnificus, C. jejuni,* and *E.coli* O157*,* have been reported to be resistant to numerous antimicrobials in the VBNC state when it comes to antibiotic stress **Ramamurthy *et al.* (2014).** According to **Pasquaroli *et al.* (2013),** staphylococcal biofilms may play a role in recurrent infections because *Staph. aureus* can enter the VBNC state in infectious biofilms and because the presence of vancomycin or quinupristin / dalfopristin may unintentionally cause a true VBNC state or persistence of *Staph. aureus* cells embedded in biofilms. Some bacterial cells may be stimulated to enter the VBNC state during the pasteurization and preservation procedures of food **Zhao *et al.* (2013); Kramer and Muranyi (2014).**VBNC cells are typically resistant to several antimicrobials, which can occasionally result in therapeutic failure **Hu and Coates (2012).** Actually, antimicrobially treated food included a significant amount of VBNC cells **Anvarian *et al.* (2016).** In addition to the decreased shelf life that leads to early food product degradation, the presence of VBNC cells is thought to pose a risk to human health as well as food safety **Ayrapetyan and Oliver (2016).** Further researches are still required to examine VBNC cell resistance to particular food treatments, such as specific processing, preservation, and packaging methods, even though it has been demonstrated that these pathogens can withstand a variety of stresses related to food safety **Ayrapetyan and Oliver (2016).**

VBNC resuscitation

The first bacterial model to illustrate resuscitation from the VBNC condition was *Salmonella enteritidis*, in which the addition of HIB (Heart Infusion Broth) allowed the bacteria to regain culture ability **Roszak *et al.* (1984).** The viable but non culturable (VBNC) cells possess an amazing ability called resuscitation, which allows them to return to normal levels of physiological and metabolic activity **Baffone *et al.* (2006).** Many techniques, such as the high dilution of the VBNC cells with artificial sea water to eliminate nutrients and prohibit the presence of culturable bacteria, are established for identifying real resuscitation and inhibit the regrowth of culturable bacteria only **Whitesides and Oliver (1997).** However, the bacteria can only be revived during a brief window of time known as the "Resuscitation Window" that follows VBNC induction. After that, there won't be any more resuscitation, and the bacteria will eventually die **Pinto *et al.* (2015).**

According to **Senoh *et al.* (2010),** the term "resuscitation" is mainly dependent on the conditions of entering and exiting the VBNC state, as well as the bacterial species and length of incubation. Certain environmental inducers, such as the removal of stress conditions, the supply of rich nutrients, the stabilization of osmotic pressure, the breakdown of hydrogen peroxide, and the presence of a host, are necessary for resuscitation from the VBNC state **Dong *et al.* (2020).**

Food-borne pathogenes

Mostly, the growing number of VBNC pathogens has a significant effect on food safety. During food preparation, transference, and storage, food is frequently exposed to a limited variety of conditions, which can present several opportunities for VBNC induction. Foodborne pathogens can be made to enter the

VBNC state by a variety of stress conditions, including freezing or refrigeration, fermentation, cooking, and the addition of additives like salt **Begley and Hill (2015).** VBNC induction in *Staph. aureus* can occur when acidic chemicals as citric and acetic acid are added during the food processing process. According to reports, *S. aureus* can be stimulated to enter the VBNC state in an acidic environment if there are enough resources present, but an acidic environment combined with food shortages can harm or even kill the bacteria **Bai *et al.*, (2019); Li *et al.*, (2020).** Moreover, various non-thermal techniques, including ultrasonic, irradiation, cold plasma technique, supercritical technologies, pulsed electrical field, high hydrostatic pressure, pulsed UV technology, and ozone are used in the sanitization of food products without heat application **Jadhav *et al.* (2021)**.

A number of studies have shown how non-thermal technologies, including electrolyzed water **Han *et al.* (2018)**, high-pressure CO2 **Zhao *et al.* (2016)**, pulsed light inactivation **Rowan *et al.* (2015)**, thermosonication **Liao *et al.* (2018)**, and non-thermal plasma **Liao *et al.* (2021)** can form the VBNC state for pathogen microorganisms. There are serious worries about food safety due to the possibility that some VBNC bacteria can maintain the expression of toxins and virulence factors **Dinu and Bach (2011).**

Food safety and Public health.

Food safety is the safeguarding of the food supply chain from contamination by microbes and chemical chemicals, and it is a global and complex issue **Gizaw (2019).** A high level of assurance (almost 100%) that a food which is ready to be consumed is free of pathogenic microorganisms and/or their toxins is necessary for safe food preparation, distribution, and storage **Rowan (1999).**

Foods, food ingredients, food contact surfaces, and the surrounding environment typically contain a wide variety of microorganisms, each with distinct growth requirements and origins. Given the remarkable diversity and dynamic nature of microorganisms, it is unsurprising that a wide variety of microbial species can be found in food, existing in various physiological states with distinct needs for survival and growth. The existence of VBNC pathogens in food and drinking water has been verified by numerous documents **Fakruddin *et al.* (2013).** The Viable but non-culturable (VBNC) bacteria have significant challenge in the context of food safety. These bacteria are alive but cannot grow on standard culture media, making them difficult to be detected and enumerated using traditional microbiological methods. This can lead to underestimation of bacterial populations in food samples and so potentially impacting food safety.

Public health has been severely impacted by the variety of the surroundings during food processing and the subsequent identification of VBNC pathogens in food. According to researches, the VBNC bacteria have the ability for resuscitating and guiding the host towards a fatal condition **Oliver and Bockian (1995).** Thus, strong evidences point to the possibility that VBNC infections cause foodborne outbreaks. An outbreak was documented in Japan in 1998 as a result of *E. coli* O157 contamination of salted salmon. It was suggested that the primary cause of the outbreak was the underestimation of viable pathogens brought on by the presence of VBNC *E. coli* **Makino *et al.*, (2000).** Another foodborne outbreak in Japan was caused by the development of VBNC *Salmonella oranienburg* after osmotic stress in dried processed squids **Asakura *et al.* (2002).** When *Escherichia coli O104:H4* strain came into contact with copper ions or tap water, it expressed genes characteristic of both Enterohemorrhagic *E. coli (EHEC)* and Enteroaggregative *E. coli (EAEC).* This led to an outbreak and multiple cases of hemolytic uremic syndrome and bloody diarrhea, ultimately entering the VBNC state **Aurass *et al.* (2011).** Furthermore, a key factor in assessing the pathogenicity of VBNC cells is the availability of circumstances that lead to bacterial resuscitation. The gastrointestinal system may be a desirable habitat for resuscitation, as evidenced by the fact that VBNC *S. typhimurium* LT4 could only recover when given orally **Amel and Amina (2008).** Unfortunately, after passing through the digestive system, *S. typhimurium* ATCC 14028 was unable to grow back into a culturable cell **Habimana *et al.* (2014).** Furthermore,

Tolba *et al.* (2020) found that *L.monocytogenes* recovered the activity of its virulence genes (in1An, prfA&hy1A) after resuscitation process. Many studies have shown that food contains VBNC cells **Rowan *et al.* (2015).**
Physiochemical properties as pH, water activity (aw), chemical composition, and disinfectant and environmental factors as high pressure CO2, high temperatures, storage time and temperature, pasteurization ,decontamination treatments, and packaging under modified atmosphere frequently expose food to a complex environmental system that acts simultaneously on contaminating bacteria leading to the VBNC state. Since these bacteria are undetectable using traditional methods, this alone poses a serious risk to public health and food safety **Fakruddin *et al.* (2013).** The ability of VBNC cells to revive within their human host increases this risk **Ayrapetyan and Oliver (2016).** Moreover, the researches have demonstrated that foodborne pathogens' VBNC cells continue to produce virulence factors **Dinu and Bach (2011).**
Briefly, Presence of VBNC bacteria in food is a matter of concern as they may still retain their pathogenic potential even though they are not actively growing. This harbor a risk of foodborne illness, if these bacteria are consumed. Additionally, VBNC bacteria could potentially resuscitate and restore their culture-ability under favorable circumstances, further complicating the assessment of the food safety.

Conclusion

Following years of study, the VBNC idea is now more widely recognized in relation to numerous foodborne infections and the adaptive mechanisms that go along with them. In certain strains, it is evident that certain circumstances, elements, and regulators during the induction and resuscitation of the VBNC state are critical Nonetheless, due to its unique secrete nature, the exact formation and resuscitation mechanisms of the VBNC state remain unclear so need more investigation. The ability of the VBNC cells to avoid discovery by traditional plate count methods, to withstand harsh environmental conditions like food pasteurization and antimicrobial agents, and to resuscitate with virulence and causing diseases represent a great threat to food safety and arising of infectious diseases.
Hence, there is an urgent need for developing quick, accurate, accessible and simple techniques for VBNC state detection. In summary, it is critical to identify innovative treatments to lower the risks associated with foodborne pathogens, prevent foodborne infections, and ensuring food safety through the potential applications of fundamental researches to detect the VBNC state.
Moreover, it is essential for food producers and regulators to implement good hygiene practices, proper sanitation procedures, and effective monitoring programs to prevent the growth and survival of VBNC bacteria in food. By understanding the mechanisms behind the VBNC state and applying appropriate control measures, the risk of foodborne illness associated with these bacteria can be minimized, contributing to overall food safety.

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